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Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF) Secretion and Cell Surface Binding Are Modulated by KDEL Receptors*

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Background: Mesencephalic astrocyte-derived neurotrophic factor is a secreted protein with an unknown mechanism of action.
Results: KDEL-like sequence of MANF ("RTDL") is required for ER retention, secretory responsiveness to ER stress, and surface binding.
Conclusion: KDEL receptors modulate secretion and surface binding of MANF.
Significance: The plasma membrane localization of KDELRs has implications for MANF and other KDEL-containing proteins.

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is an endoplasmic reticulum (ER) stress-responsive protein with neuroprotective effects in animal models of neurodegeneration, but the underlying mechanism is not understood. We constructed a set of lentiviral vectors that contain or lack the highly conserved final four amino acids of MANF ("RTDL"), which resemble the canonical ER retention signal ("KDEL"), to study MANF regulation in neuroblastoma cells and rat primary cortical neurons. The RTDL sequence was required for both ER retention and secretory response to ER stress. Overexpression of KDEL receptor paralogs (KDELRs) differentially reduced MANF secretion but had no effect on MANF lacking RTDL. MANF binding to the plasma membrane also required the RTDL sequence and was inhibited with a peptide known to interact with KDELRs, suggesting MANF binds KDELRs at the surface. We detected surface localization of FLAG-tagged KDELRs, with levels increasing following ER stress. Our study provides new insight into the regulation of MANF trafficking and has implications for other secreted proteins containing a KDEL-like retention signal.

Neurotrophic factors (NTFs)[2] are secreted proteins that activate signaling pathways in neurons by binding to surface receptors. NTFs promote a variety of neuronal processes, including survival, migration, regeneration, differentiation, and synaptic plasticity. The ability of NTFs to alter viability of neuronal tissue makes them candidates for treating neurodegenerative diseases. Mesencephalic astrocyte-derived neurotrophic factor (MANF), originally identified in an *in vitro* screen for secreted proteins with pro-dopaminergic properties (1), has been shown to act as an NTF, but the mechanism of action is not known. In *Drosophila*, loss of the MANF ortholog leads to a deficiency in dopaminergic neuron development, which can be rescued by fly and human MANF (2). In the rat 6-hydroxydopamine model of Parkinson disease, injection of recombinant MANF protein into the striatum reduced degeneration of dopaminergic neurons and improved behavioral outcomes (3). We previously reported that MANF is also protective against ischemic injury in a rat stroke model. When administered prior to middle cerebral artery occlusion, recombinant MANF protein (4) or transduction by an adeno-associated virus encoding MANF (5) reduced infarct volume and improved behavioral recovery.

The protective effects of recombinant MANF protein, when delivered directly into the brain, suggest an extracellular site of action, but no cognate surface receptor has been identified. Several lines of evidence also point toward intracellular functions of MANF. A recent study demonstrated a high degree of structural homology between the C-terminal domain of MANF and Ku70 (6), an inhibitor of Bax-dependent apoptosis (7). Hellman *et al.* (6) demonstrated that cytoplasmic injection of recombinant MANF protects neurons from Bax-mediated cell death; however, as MANF localizes to the ER lumen (8, 9), it is currently unclear whether endogenous MANF functions in the cytoplasm. Additional clues to the function of MANF come from studies reporting the responsiveness of MANF expression and secretion to ER stress. Stress-induced transcriptional upregulation is driven by a type 2 ER stress-response element in the MANF promoter (10), and increased secretion was observed in HEK293, HeLa, and cardiomyocytes in response to ER stress-inducing molecules (8, 9, 11).

MANF secretion is influenced by its N- and C-terminal sequences. The first 21 residues function as a signal peptide, which direct MANF to the ER during protein synthesis and allow access to the secretory pathway (12). At the extreme C

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[2] The abbreviations used are: NTF, neurotrophic factor; BFA, brefeldin A; BGS, bovine growth serum; ER, endoplasmic reticulum; KDELR, Lys-Asp-Glu-Leu endoplasmic reticulum protein retention receptor; MANF, mesencephalic astrocyte-derived neurotrophic factor; SP, signal peptide; Tg, thapsigargin; DIV, day *in vitro*; oligo, oligonucleotide.

terminus is the sequence RTDL, which resembles the canonical ER retention signal (KDEL). MANF is the only protein in the human proteome known to contain these four C-terminal amino acids (query of *Homo sapiens* RefSeqs). It has been speculated that an interaction with a Lys-Asp-Glu-Leu (KDEL) endoplasmic reticulum protein retention receptor (KDELR) could regulate MANF trafficking and secretion (13). In support of this hypothesis, elevated secretion of murine MANF was detected upon removal of the RTDL sequence (14). Although this finding suggests MANF trafficking is regulated by the KDELRs, an interaction between MANF and a KDELR has not been reported, and the relationship between MANF and KDELRs under conditions of ER stress is not understood.

The human genome contains three KDELR genes (*KDELR1*, *KDELR2*, and *KDELR3*). Each of the KDELRs functions in the Golgi by binding to proteins that contain an ER retention signal on their C terminus and facilitating their retrograde trafficking to the ER. The functional differences between the paralogs are currently unclear (15). Although a C-terminal KDEL sequence is the canonical retention signal, the KDELRs also have some affinity for similar sequences. An *in vitro* screen examining the ability of short peptides to interact with the KDELRs revealed 40–80% percent binding of the RTDL sequence compared with the canonical KDEL sequence (16). It is possible that differences in affinity for the KDELR are important for MANF secretion, and a competition model for ER retention could explain the rapid secretion of MANF in response to ER stress.

The secretion of a protein containing a KDELR-interacting sequence is not unprecedented. Additional proteins with ER retention signals are also detected extracellularly, including GRP78 (BiP) and GRP94 (Hsp90β), and there is evidence for the function of these proteins in the extracellular space. For example, GRP78 was first identified at the surface of malignant lymphocytes (17), and the elevated levels observed on the surface of malignant cells have been exploited to successfully target and kill these cells with small molecules (18, 19). GRP94 is similarly detected at the membrane in the nervous system, where it was found to regulate cell migration (20).

Although it is widely accepted that MANF is secreted, an attempt to detect an interaction of ^{125}I-labeled MANF with the plasma membrane of sympathetic neurons was not successful (6). The purpose of this study was to examine the functions of the RTDL sequence in MANF secretion and surface binding in neuronal cell types. We observed that the RTDL sequence is essential for MANF localization and rapid secretory response to thapsigargin (Tg)-induced ER stress, and our studies provide support for a direct interaction between the RTDL sequence of MANF and KDEL receptors. This is the first study to demonstrate MANF binding to the cell surface, and we show that the C-terminal RTDL sequence is required for this event. Furthermore, we propose that surface binding is modulated by the KDEL receptor. Consistent with a direct interaction between these two proteins at the membrane, we detected overexpressed KDELRs at the surface of SH-SY5Y cells, observed competitive inhibition of surface MANF with a KDELR-interacting peptide, and observed an increase in surface MANF following KDELR1 overexpression. The relationship between MANF and the KDELRs, both intra- and extracellularly, may serve to confer its protective actions.

EXPERIMENTAL PROCEDURES

Plasmids—The human MANF open reading frame (ORF) was amplified from pdsAAV CMV human MANF using attB-linked oligos and recombined into pDONR221 donor vector using a standard BP reaction (Invitrogen). The coding sequence for enhanced GFP (Clontech) was amplified using attB-linked oligos and cloned into the EagI site of the pDONR221-MANF entry vector to produce pDONR221-GFP-MANF using Infusion (Clontech). Next, a stop codon was inserted immediately after the BstAPI site to produce pDONR221-GFP-MANF-ΔRTDL. The pDONR221-SP-GFP entry vector containing the MANF signal peptide fused to enhanced GFP was produced by replacing the EagI-RsrII fragment of pDONR221-MANF with the coding region and endogenous stop codon of enhanced GFP. The pDONR221-SP-GFP-RTDL entry vector was produced by amplifying the SP-GFP open reading frame (from pLenti6.3-SP-GFP) using attB-linked oligos, with the reverse oligo including the nucleotides encoding the ASARTDL sequence. All entry vectors were sequence-verified and maintained in the bacterial strain top10 (Invitrogen) prior to recombination into the pLenti6.3-V5/DEST destination vector using a standard LR reaction (Invitrogen). All plasmids based on LV backbones were maintained in the recombination-deficient bacterial stain Stbl3 (Invitrogen). The DsRed-ER plasmid was purchased from Clontech, and the Myc-FLAG-tagged human KDELRs were purchased from Origene (KDELR/accession numbers KDELR1/NM_006801.2, KDELR2/NM_006854.2, KDELR3a/NM_006855.2, and KDELR3b/NM_016657.1).

Lentivirus—The pLenti6.3-based MANF expression vectors were packaged into virions using the ViraPower packaging mixture (Invitrogen) following the manufacturer's protocol. Viral particles were concentrated by ultracentrifugation on a sucrose cushion (21) and resuspended in 1× Hanks' buffered saline solution.

Antibodies and Other Reagents—Affinity-purified rabbit polyclonal MANF antibody was produced by Yenzyme Antibodies using a peptide corresponding to amino acids 160–182 of human MANF. Additional antibodies were monoclonal anti-actin (Abcam, clone C4), polyclonal anti-actin (Sigma), monoclonal anti-GFP (Roche Applied Science), monoclonal anti-FLAG (Sigma, clone M2), polyclonal anti-protein-disulfide isomerase (Cell Signaling), and polyclonal anti-RCAS1 (Cell Signaling). For peptide competition experiments, reverse phase HPLC-purified peptides (The Johns Hopkins University Synthesis and Sequencing Facility, Baltimore, MD) were suspended in sterile water or cell culture medium at 10 mM. Thapsigargin (Sigma) was suspended in DMSO at 1 mM and brefeldin A (Sigma) in ethanol at 5 mg/ml. Vehicle controls (at concentrations equivalent to treatments) were used in all experiments.

Primary Cortical Neurons—Rat primary cortical cultures were prepared as described previously (22) and in accordance with approved procedures by the National Institutes of Health Animal Care and Usage Committee. Cells were plated at 6×10^4 and 1×10^6 viable cells/well in 96- and 6-well polyethyleneimine-coated plates, respectively. Cells were fed by 50%

media exchange starting on the 4th day *in vitro* (DIV4). Additional feedings were conducted on DIV6, -8, -11, and -13.

Tissue Culture—SH-SY5Y cells were maintained in DMEM (4.5 g/liter glucose, 110 mg/ml sodium pyruvate) supplemented with 10% BGS (Hyclone), 10 units/ml penicillin, and 10 μg/ml streptomycin. HEK293 cells were maintained in the same medium as above, with 5% BGS. For stable cell lines, SH-SY5Y cells were transduced with serial dilutions of the concentrated lentivirus, and stable integrants were selected on 2 μg/ml blasticidin for 2 weeks in a 24-well plate. Colonies expressing GFP at visibly detectable levels were pooled into a heterogeneous population and expanded for experimentation. Cells were grown in a humidified incubator at 37 °C with 5.5% CO_2. For transient transfections, SH-SY5Y and HEK293 cells were transfected in normal growth medium with 0.4 μg of DNA and 0.12 μl of Xfect (Clontech) per cm^2. Complexes were removed from the cells after 4 h and replaced with complete media. Transfections were allowed to proceed for 48 h before experimental assays were continued, unless otherwise noted.

Measurement of GFP-MANF Fluorescence—Cells were grown in phenol red-free medium (as described above). Medium was collected and centrifuged for 10 min at 3000 $\times$ *g* (at 4 °C) to pellet nonadherent cells and debris. Supernatant was loaded onto 96-well black-walled plates, and GFP fluorescence was measured in Biotek Synergy 2 plate reader using excitation at 485/20 and emission at 528/20 filters (BioTek). Background fluorescence was measured in media not exposed to cells, and the average background was subtracted from experimental measurements.

Immunoprecipitation of Secreted GFP-MANF—Stable SH-SY5Y cells were plated at 8.0×10^5 cells per well in 9.5-cm^2 dishes (Corning Glass). Cell culture medium was collected after 48 h and spun at 3000 $\times$ *g* for 10 min (4 °C) to pellet dead cells. To account for differences in total cell number, the amount of supernatant entering the immunoprecipitation was determined using protein concentrations of the whole cell lysates. Anti-GFP-conjugated beads (MBL International, clone RQ2) or nonspecific control beads were added and rotated overnight at 4 °C. For MANF, 5 μg of antibody (Yenzyme) or control (5 μg of anti-FLAG) was added to the medium for 2 h at 4 °C. Protein G-Sepharose beads were then added and rotated overnight at 4 °C. Samples were washed three times with 1 ml of wash buffer containing 50 mM Tris-HCl (pH 7.2), 250 mM NaCl, 2 mM EDTA, 10% glycerol, 0.1% Nonidet P-40 (Pierce), 1$\times$ protease inhibitor mixture (Sigma). Samples were eluted for 10 min at 70 °C in 2$\times$ LDS buffer containing 286 mM β-mercaptoethanol.

Western Blots—Cells were lysed in a modified RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 1$\times$ protease inhibitor mixture. Samples were quantified with a DC assay (Bio-Rad), and equal amounts of total protein were separated on 4–12% NuPAGE gels using MOPS running buffer. Proteins were transferred to 0.20-μm PVDF membranes (Invitrogen) and immunoblotted using LI-COR reagents (LI-COR Biosciences). Secondary antibodies were IR700 and IR800 (Rockland Immunochemicals), and blots were scanned using an Odyssey scanner (LI-COR).

Live Cell Imaging—SH-SY5Y cells were imaged in standard culture medium (as described above) using a Nikon Eclipse TE2000-E inverted microscope equipped with a Nikon $\times$20 (0.45 NA) Plan Fluor objective and Nikon Digital Sight DS-QiMc camera. Images were acquired with Nikon Elements AR software and processed using Adobe Photoshop.

Immunocytochemistry and Confocal Microscopy—Cells were seeded at 1.0×10^5 (immunocytochemistry) or 8.0×10^5 (transient transfections) cells in 35-mm dishes containing 25-mm round glass coverslips (Warner Instruments). For GFP fluorescence, cells were transfected for 24 h, fixed with 4% paraformaldehyde in PBS (pH 7.4), and stained with 1 μg/ml DAPI (in PBS) for 15 min. For immunocytochemical staining, cells were fixed 24 h after seeding, permeabilized for 30 min at room temperature (PBS + 0.1% Triton X-100, 0.2% BSA), and blocked for 1 h at room temperature (PBS + 0.1% Triton-X100, 2% BSA, 5% normal goat serum). Primary antibodies were added to the coverslips (in PBS + 5% goat serum) and incubated with gentle rotation overnight at 4 °C. After washing with PBS, Alexa Fluor secondary antibodies (Invitrogen) were added for 1 h at room temperature, and nuclei were stained with 1 μg/ml DAPI (in PBS). Coverslips were mounted to glass slides with Mowiol 4–88 (EMD) and allowed to set overnight. A Nikon Eclipse-C1 confocal microscope equipped with a Nikon $\times$100 (1.30 NA) PlanFluor objective was used to image samples. Nikon EZ-C1 software was used for image acquisition, and images were processed in Adobe Photoshop.

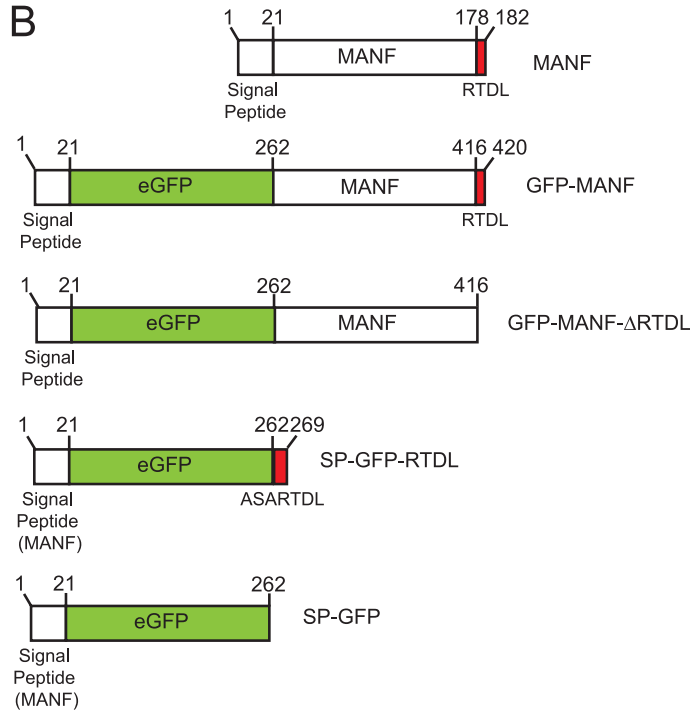
Surface Biotinylation—SH-SY5Y cells were plated at 2.8×10^6 cells per 60-mm dish and allowed to grow overnight. HEK293 cells were plated at 1.0×10^6 cells per well of a 6-well plate and allowed to grow overnight before transient transfection. Primary cortical neurons were plated at 1.0×10^6 cells per well of 6-well plate and allowed to grow for 14 days. Cells were cooled on ice, washed twice with ice-cold PBS, and labeled for 20 min with cold Sulfo-NHS-SS-Biotin solution (0.25 mg/ml in PBS) at 4 °C. The biotinylation reaction was quenched for 5 min at 4 °C, and the cells washed twice with cold PBS. Cells were lysed in RIPA buffer and quantified by DC assay. Input into the streptavidin pulldown was determined based on the total protein concentration. Pulldowns were performed overnight at 4 °C with end-over-end rotation. Samples were washed three times with 1 ml of wash buffer containing 25 mM Tris, 150 mM NaCl, 0.2% Nonidet P-40, 0.1% SDS, and protease inhibitors. Samples were eluted for 1 h at room temperature in 2$\times$ LDS buffer containing 50 mM DTT.

RESULTS

GFP-tagged MANF Is Localized to the ER—The MANF protein is highly evolutionarily conserved, with 97.8% identity between the human and mouse protein sequences (Fig. 1*A*). Focusing on the final four amino acids of MANF, there was an apparent divergence from the canonical ER retention sequence (KDEL) moving from invertebrates to vertebrates. Based on the ER and Golgi localization of MANF (8, 9), we hypothesized that the RTDL sequence of human MANF functions as an ER retention signal critical to its trafficking. To better understand the role of this sequence in MANF localization in cells of neuronal origin, we created a set of lentiviral expression vectors encoding

A

Species	Carboxy-terminus	% Identity (total protein)
Homo sapiens	-R T D L	
Pan troglodytes	-R T D L	98.9
Canis familiaris	-R T D L	94.4
Bos taurus	-R T D L	95.5
Rattus norvegicus	-R T D L	97.8
Mus musculus	-R T D L	97.8
Danio rerio	-R T D L	70.7
Drosophila melanogaster	-R S E L	55.3
Anopheles gambiae	-K T E L	54.7
Caenorhabditis elegans	-K E E L	50.3
Canonical ER retention	-K D E L	



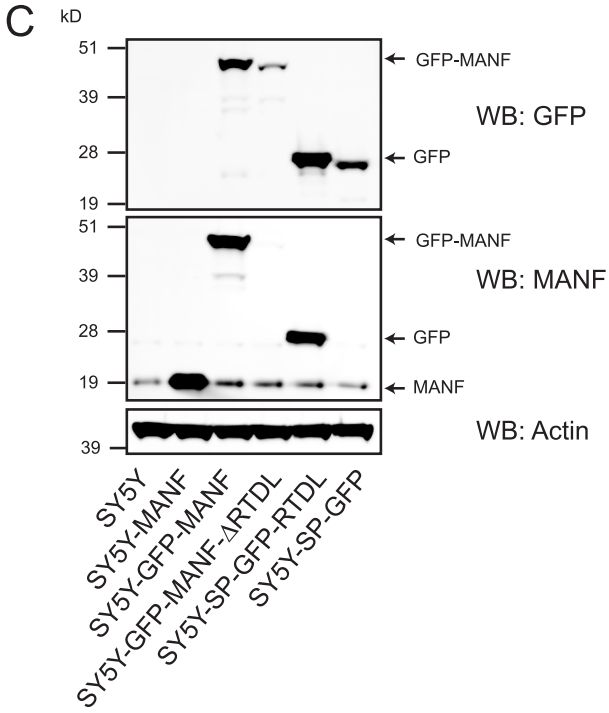


FIGURE 1. **Stable SH-SY5Y cells expressing MANF variants were created to study the function of the C-terminal RTDL sequence.** *A,* examining orthologous MANF protein sequences reveals a divergence away from the canonical ER retention sequence for the final four amino acids. Negatively (*red*) and positively (*blue*) charged amino acids are indicated. *B,* various combinations of human MANF coding sequences were cloned into a lentiviral backbone. *C,* stable SH-SY5Y cell lines were transduced with lentivirus, and protein expression was analyzed by immunoblotting for GFP and MANF. Note that the MANF antibody was generated using a peptide corresponding to the C terminus of the MANF protein (which includes the ASA-RTDL sequence) and thus lacks affinity for ΔRTDL protein and detects SP-GFP-RTDL. Actin was used as a loading control. *WB*, Western blot.

MANF and MANF lacking RTDL (ΔRTDL), both as untagged and GFP-tagged variants (Fig. 1*B*). For GFP-tagged constructs, the signal peptide (SP) of MANF (amino acids 1–21) was placed upstream of the GFP coding sequence to preserve ER targeting and translocation. GFP-tagged control constructs (SP-GFP-RTDL and SP-GFP) contain only the signal peptide and the final seven amino acids of MANF (ASA-RTDL) and served to examine the function of the terminal sequences independent of additional MANF sequence. The three residues upstream of RTDL (ASA) were included at the C-terminal end of GFP, as the −5 and −6 positions of the KDEL C terminus were previously identified as important for ER retention (23).

The neuroblastoma cell line, SH-SY5Y, was transduced with lentivirus to create stable cell lines expressing the MANF and GFP variants. Expression of the exogenous proteins in the stable cell lines was analyzed by Western blot, which revealed proteins of the expected molecular weights (Fig. 1*C*). An anti-MANF antibody was used to assess endogenous and untagged MANF. The anti-MANF antibody recognizes a C-terminal epitope of the MANF protein, which includes the ASA-RTDL sequence, and thus lacks affinity for ΔRTDL protein; therefore, it was necessary to use an anti-GFP antibody to compare expression of GFP-MANF to GFP-MANF-ΔRTDL. Conversely, the ASA-RTDL added to the SP-GFP-RTDL fusion had sufficient affinity with the MANF antibody to be detected by immunoblotting.

Next, we compared the intracellular localization of endogenous MANF to the GFP-MANF fusion protein in SH-SY5Y cells. Endogenous MANF, which was previously reported to localize to the ER in U20S cells and cardiac myocytes (8, 9), was first examined in the parental SH-SY5Y cells (Fig. 2*A*). Cells were transiently transfected with a fluorescent ER marker (DsRed-ER) and immunostained with a MANF antibody. Confocal microscopy revealed extensive overlap between the two signals, consistent with ER localization of the protein in SH-SY5Y cells (Fig. 2*A*). Next, we examined GFP-MANF in the stable SH-SY5Y cell line, and we detected overlap of the GFP signal with DsRed-ER, indicating successful targeting of the fusion protein to the ER (Fig. 2*B*). Colocalization of GFP-MANF with other cellular compartments, including the Golgi, nucleus, and mitochondria, was also examined. GFP-MANF fluorescence did not overlap with immunofluorescence from RCAS1 (Fig. 2*C*), a transmembrane protein localized predominantly to the Golgi (24), the nucleus (Fig. 2*C*, *DAPI*), or the inner mitochondrial membrane protein COX IV (data not shown). These results demonstrate that GFP-MANF, like endogenous MANF, is localized primarily to the ER in SH-SY5Y cells.

To examine the role of the RTDL sequence in mediating ER localization, we examined SH-SY5Y cells stably expressing SP-GFP-RTDL. This protein also colocalized with DsRed-ER, consistent with the RTDL sequence functioning as an ER retention signal independent of additional MANF sequences (Fig. 2*D*). Similar to GFP-MANF, SP-GFP-RTDL did not overlap with RCAS1 or DAPI (Fig. 2*E*).

C-terminal RTDL Sequence Is Critical for MANF Localization—Using epifluorescence microscopy, SH-SY5Y cells stably expressing GFP-MANF-ΔRTDL exhibited less intracellular fluorescence compared with cells stably expressing full-length GFP-MANF (Fig. 3*A*). This finding suggested an increase in secretion of the GFP-MANF protein lacking RTDL. To test the relative levels of secreted GFP-MANF and GFP-MANF-ΔRTDL, we directly measured fluorescence of the media with GFP optimized filters. We observed increased fluorescence in the medium collected from SY5Y-GFP-MANF-ΔRTDL cells compared with SY5Y-GFP-MANF cells (Fig. 3*B*). Our approach of measuring fluorescence in the medium to approximate relative GFP-MANF levels was validated in two ways. First, fluorescence signal was reduced when the media were immunoprecipitated using GFP or MANF antibodies (Fig. 3*C*). Second, we observed a linear relationship between fluorescence in the media and the relative GFP-MANF concentration, allowing us to infer relative levels of the proteins using this assay (Fig. 3*D*). As an alternative approach to evaluate secretion, we immunoprecipitated GFP-MANF and GFP-MANF-ΔRTDL from cell culture medium and compared intracellular and secreted protein levels by immunoblot. This approach confirmed increased GFP-MANF-ΔRTDL in the media compared with GFP-MANF (Fig. 3, *E* and *F*).

GFP-MANF-ΔRTDL Localizes to the ER and Golgi and Accumulates in the ER after Brefeldin A Treatment—To further characterize GFP-MANF-ΔRTDL, we investigated the subcellular localization of the intracellular protein by confocal microscopy. We observed an overlap between GFP-MANF-ΔRTDL and DsRed-ER localization, similar to what was observed for wild-type MANF, albeit at reduced intracellular levels (Fig. 4*A*). GFP-MANF-ΔRTDL also did not colocalize with the mitochondria or nucleus (Fig. 4*A*, *DAPI*). In contrast to wild-type MANF, we observed large perinuclear accumulations of GFP-MANF-ΔRTDL (Fig. 4*A*, *top panel, white arrows*). We hypothesized that this accumulation was localized to a post-ER compartment of the secretory pathway. To test the possibility of Golgi localization, the cells were immunostained with anti-RCAS1. Confocal microscopy revealed extensive overlap between the perinuclear accumulations of GFP-MANF-ΔRTDL and RCAS1 (Fig. 4*A*, *bottom panel, white arrow*). The subcellular localization of SP-GFP was also examined in the stably transduced SH-SY5Y cell line. Like GFP-MANF-ΔRTDL, we observed an accumulation of SP-GFP in the Golgi (Fig. 4*B*), supporting similar trafficking of the two proteins.

The localization of GFP-MANF-ΔRTDL to the ER and Golgi is consistent with GFP-MANF following the classical secretory pathway in SH-SY5Y cells. To confirm this pathway of secretion, we treated cells with BFA, an inhibitor of ER to Golgi transport (25). We utilized live cell imaging to monitor protein localization and observed an accumulation of intracellular fluorescence for the SY5Y-GFP-MANF-ΔRTDL cells treated over 8 h with 50 ng/ml BFA (Fig. 4*C*). We did not detect a similar effect for SY5Y-GFP-MANF cells, consistent with limited secretion of the wild-type protein during this time frame. The BFA-accumulated GFP-MANF-ΔRTDL colocalized with DsRed-ER when examined by confocal microscopy (Fig. 4*D*). Overall, these observations provide evidence for increased secretion, through the classical secretory pathway, for GFP-MANF lacking the RTDL sequence.

RTDL Sequence Mediates Secretory Responsiveness to Thapsigargin-induced ER Stress—MANF secretion is highly responsive to ER stress in HEK293, HeLa, and cardiomyocytes (8, 9, 11). These studies used Tg, a compound that causes ER stress by inhibiting the ER Ca^{2+}-ATPase and depleting ER Ca^{2+} levels (26). We treated the SY5Y-GFP-MANF cell line with Tg for 5 h and detected a decrease in intracellular fluorescence by microscopy, suggestive of elevated secretion (Fig. 5*A*). To characterize the role of the RTDL motif in this Tg stress model, we compared the secretion of GFP-MANF to GFP-MANF-ΔRTDL into culture medium over time in the presence or absence of 300 nM Tg (Fig. 5*B*). In SY5Y-GFP-MANF cells treated for 5 h with thapsigargin, we observed a significant increase in fluores-

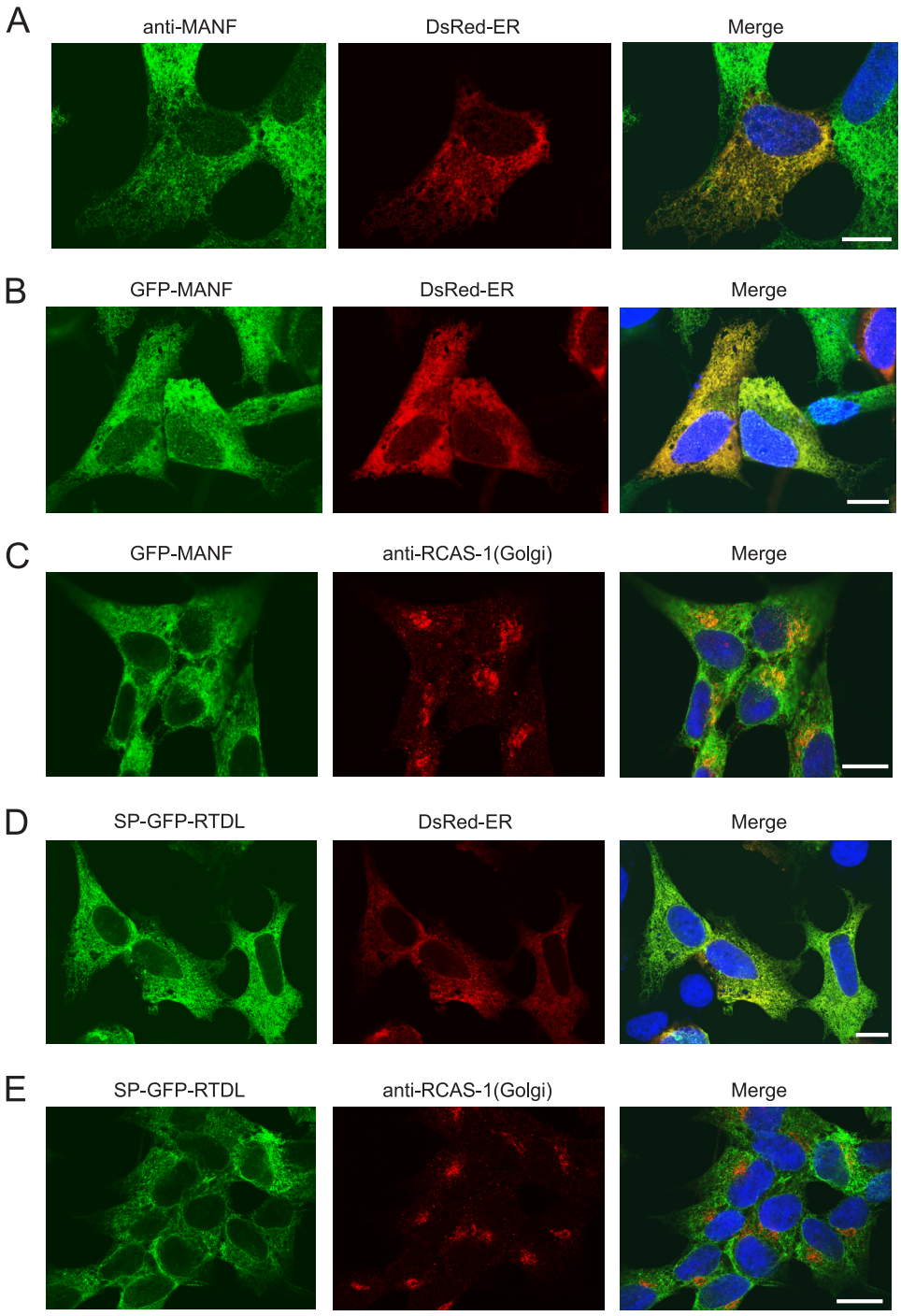


FIGURE 2. **MANF and GFP-tagged MANF localize to the ER in SH-SY5Y cells.** *A,* endogenous MANF colocalizes with an ER-targeted fluorescent protein in SH-SY5Y cells. MANF localization was examined by immunocytochemistry and confocal microscopy. Cells were transiently transfected for 24 h with DsRed-ER and immunostained with a MANF antibody. Nuclei were stained with DAPI. *B,* GFP-MANF localizes to the ER in SH-SY5Y cells. Cells stably expressing GFP-MANF were transiently transfected with DsRed-ER for 24 h and examined by confocal microscopy. *C,* GFP-MANF does not show extensive overlap with the Golgi. Stable SH-SY5Y-GFP-MANF cells were immunostained with anti-RCAS1 to identify the Golgi. *D,* SP-GFP-RTDL localizes to the ER. Stable cells were examined as described in *B*. *E,* SP-GFP-RTDL does not colocalize with RCAS1. Stable cells were examined as described in *C*. *Scale bars* on micrographs = 10 μm.

cence (2.2 ± 0.1-fold, $p < 0.05$), whereas SY5Y-GFP-MANF-ΔRTDL cells showed a decrease (0.92 ± 0.001-fold, $p < 0.05$), compared with their respective vehicle controls. This result supports an RTDL-dependent induction of MANF secretion in response to thapsigargin-induced ER stress. Increased secretion, however, was not observed when ER stress was induced with the glycosylation inhibitor tunicamycin (Fig. 5*C*) or the reducing agent dithiothreitol (data not shown). Both of these compounds induce protein misfolding, and the lack of secretory response suggests GFP-MANF secretion is not a general response to all types of ER stress.

We next examined SP-GFP-RTDL and SP-GFP to test the possibility that the RTDL sequence of MANF is sufficient for secretory response to thapsigargin. Following 5 h of 300 nM Tg

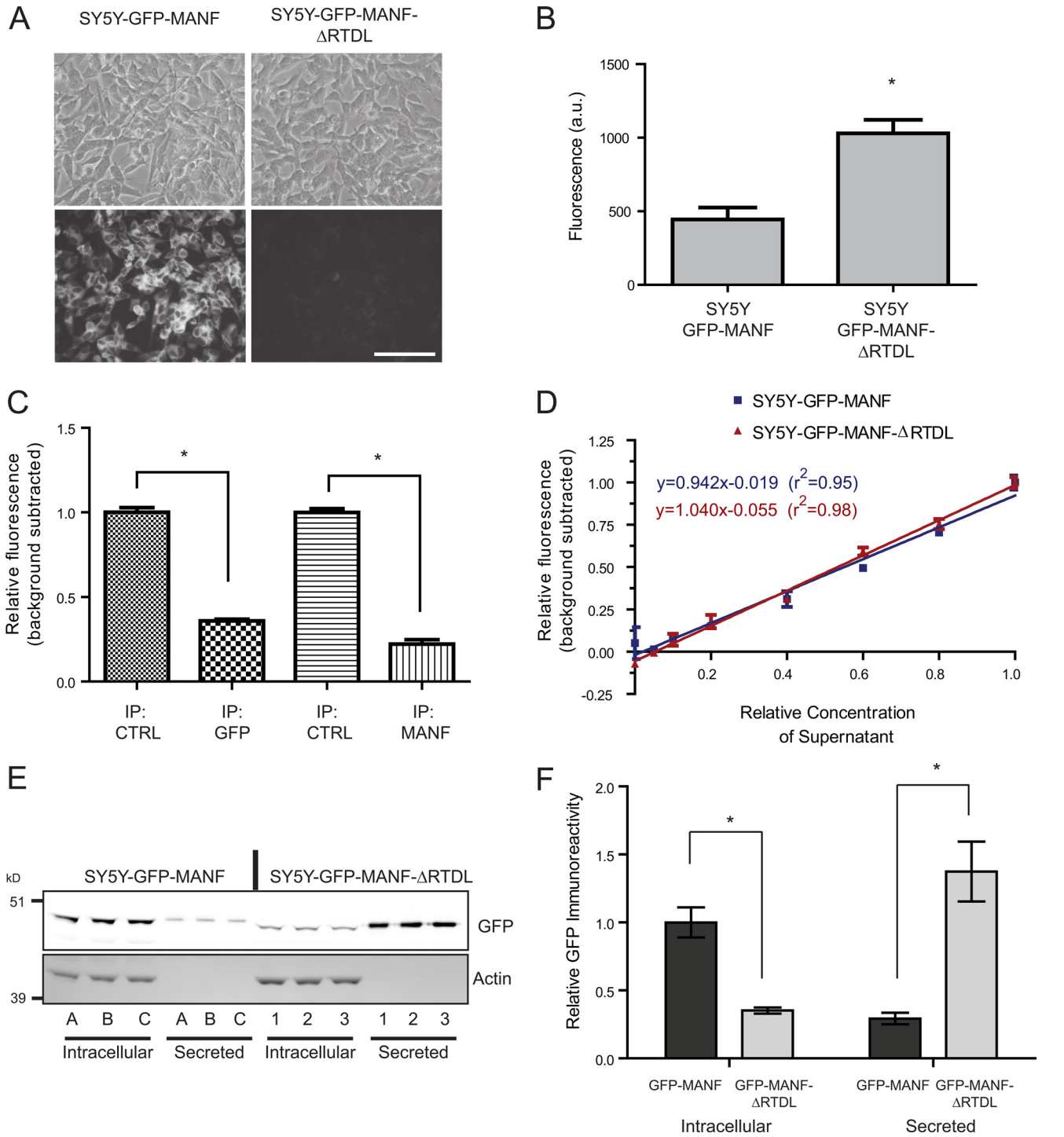
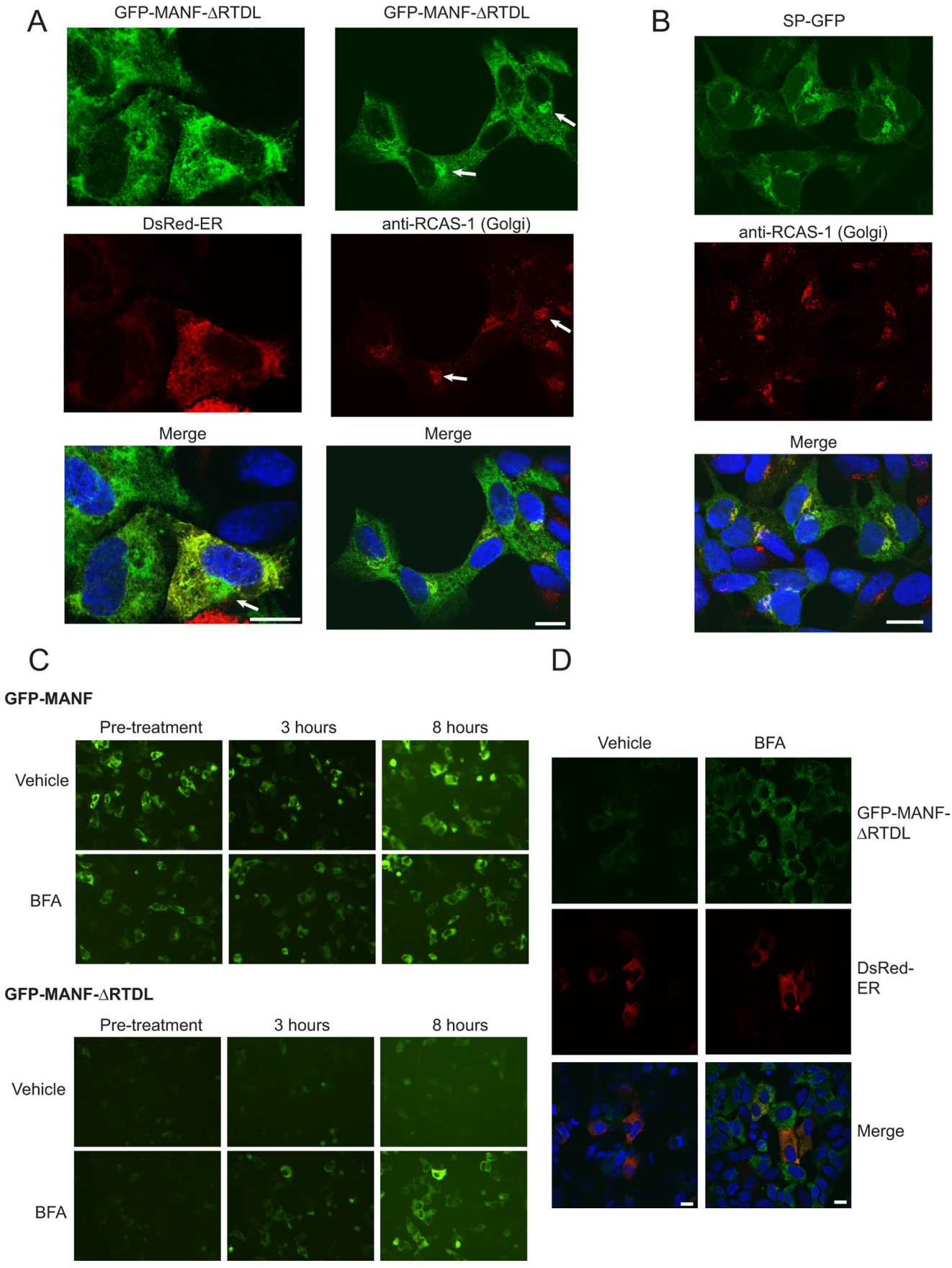


FIGURE 3. **Removal of the C-terminal RTDL sequence from GFP-MANF increases its secretion.** *A,* SH-SY5Y cells stably expressing GFP-MANF or GFP-MANF-ΔRTDL were examined by microscopy for fluorescence. Images were captured using identical exposure settings. *Scale bar,* 50 μm. *B,* culture medium from GFP-MANF-ΔRTDL has increased fluorescence compared with GFP-MANF cells ($p < 0.0001$, two-tailed *t* test). GFP fluorescence measured in cell culture medium (mean ± S.D., $n = 6$) was normalized to total protein lysate, as a proxy for cell number. *a.u.,* arbitrary units. *C,* fluorescence in medium collected from SY5Y-GFP-MANF cells was measured before and after anti-GFP or anti-MANF immunoprecipitation (*IP*) ($n = 3$). Control (*CTRL*) agarose (matching anti-GFP bead composition) or Sepharose beads conjugated to anti-FLAG (anti-MANF control) were used as controls. Anti-GFP reduced fluorescence 64 ± 3%, and anti-MANF reduced fluorescence 78 ± 3%, *t* test, $p < 0.0001$). *D,* cell culture supernatant from SY5Y-GFP-MANF and SY5Y-GFP-MANF-ΔRTDL cells was diluted with blank medium and measured for fluorescence. A best fit line for the data was modeled using linear regression. Analysis of covariance was used to test for differences in the slopes of the lines. The slopes did not differ significantly between the two cell lines, $F(1,44) = 3.24$, $p = 0.08$. *E,* increased GFP-MANF-ΔRTDL is detected in cell culture medium by immunoprecipitation. Secreted MANF was immunoprecipitated from the cell culture media with anti-GFP beads. Intracellular and secreted MANF protein levels were compared by immunoblotting for GFP or MANF. Actin served as a loading control for intracellular proteins. *F,* densitometry of *E*. *, $p < 0.01$ (two tailed *t* test).


A
GFP-MANF-ΔRTDL
GFP-MANF-ΔRTDL
DsRed-ER
anti-RCAS-1 (Golgi)
Merge
Merge
B
SP-GFP
anti-RCAS-1 (Golgi)
Merge
C
GFP-MANF
Pre-treatment
3 hours
8 hours
Vehicle
BFA
GFP-MANF-ΔRTDL
Pre-treatment
3 hours
8 hours
Vehicle
BFA
D
Vehicle
BFA
GFP-MANF-ΔRTDL
DsRed-ER
Merge

treatment, we detected elevated secretion of GFP-MANF (6.6 ± 0.13-fold, Tg *versus* untreated, $p < 0.0001$) and SP-GFP-RTDL (5.9 ± 0.20-fold, Tg *versus* untreated $p < 0.0001$), whereas secretion of GFP-MANF-ΔRTDL (0.78 ± 0.04, $p < 0.01$) and SP-GFP (0.73 ± 0.09, $p < 0.01$) both decreased (Fig. 5*D*). Comparison of intracellular and extracellular MANF ratios by immunoprecipitation and Western blot analysis also revealed a shift toward secretion for both GFP-MANF and SP-GFP-RTDL, although the effects observed using this approach were less pronounced than with measurements of media fluorescence (Fig. 5*E*).

KDELRs Regulate MANF Secretion—Our results demonstrate that the RTDL sequence of MANF is important for mediating ER localization and regulating the thapsigargin-induced secretion in SH-SY5Y cells. These results support an interaction between the RTDL sequence and KDELRs. To directly assess the role of the KDELRs in MANF localization, we overexpressed four human KDELR gene products as Myc-FLAG-tagged fusion proteins. Localization of the tagged receptor was assessed with an anti-FLAG antibody on transfected cells. We observed a strong signal localized to the Golgi for all four receptor isoforms, consistent with the established location of KDELRs (Fig. 6*A*).

To determine the effect of KDELR overexpression on GFP-MANF secretion, we transiently overexpressed the KDELRs in the SH-SY5Y cells stably expressing GFP-MANF or GFP-MANF-ΔRTDL and measured fluorescence in the medium. For GFP-MANF-expressing cells, we observed a significant percent decrease in media fluorescence when overexpressing KDELR1 or KDELR2 but no change for KDELR3a or KDELR3b (Fig. 6*B*). As predicted, KDELR overexpression did not have any effect on GFP-MANF-ΔRTDL secretion. We next examined the effects of KDELR overexpression under conditions of ER stress, which increase secretion of GFP-MANF compared with basal conditions (Fig. 6*C*). After 5 h of 300 nM Tg treatment, we observed a significant decrease in MANF secretion for cells overexpressing KDELR2, KDELR3a, or KDELR3b but no significant effect for KDELR1. For GFP-MANF-ΔRTDL, we again detected a decrease in secretion in response to Tg but no change related to overexpression of the KDELRs. We also examined the localization of GFP-MANF and the KDELRs following Tg stress, and we detected colocalization of the proteins (Fig. 6*D*). These observations are consistent with the KDEL receptors interacting with the RTDL sequence of MANF and facilitating intracellular retention. The data also indicate that the KDELR isoforms have nonoverlapping roles under different stress conditions.

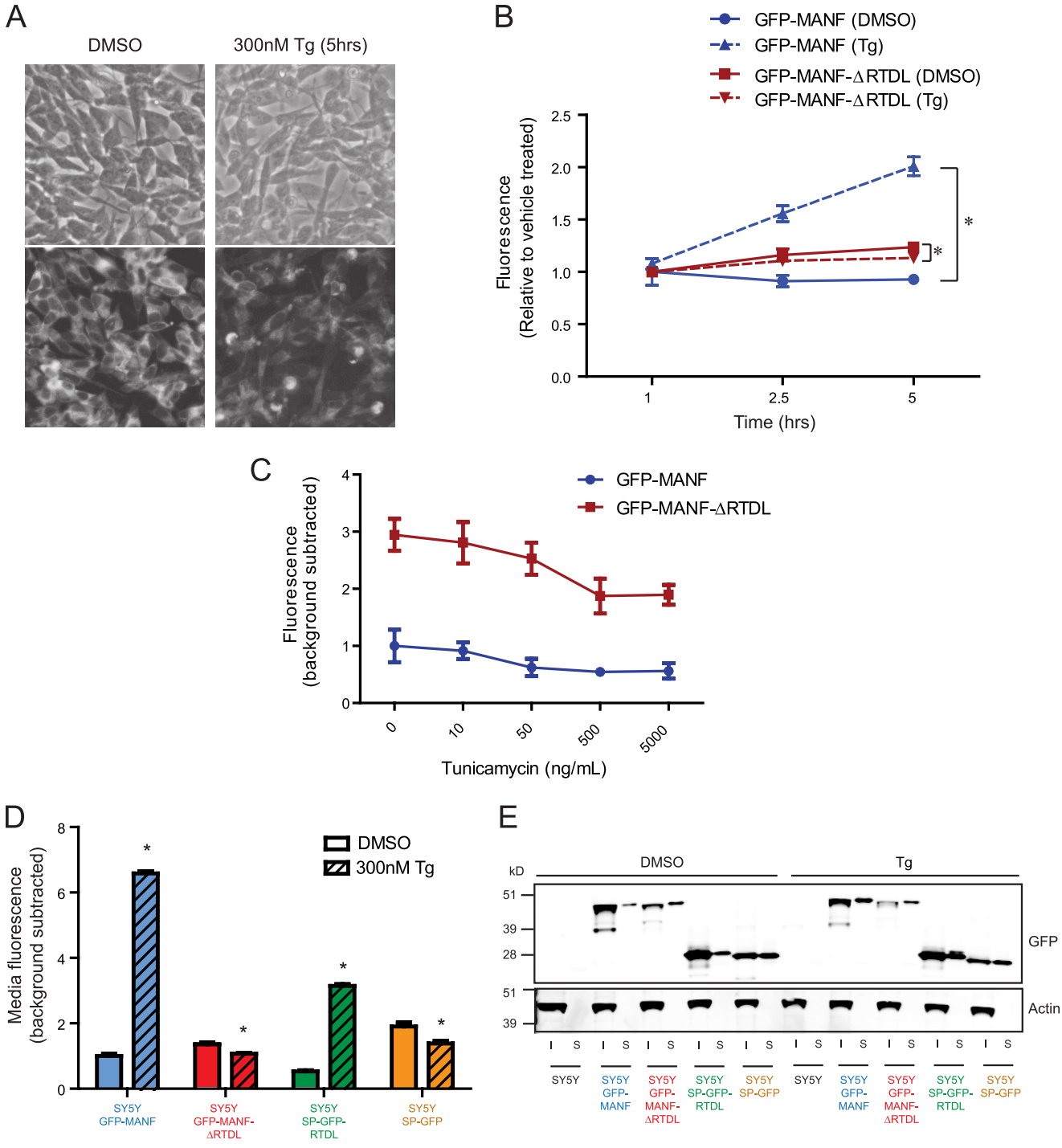
MANF Binding to the Cell Surface Requires the RTDL Sequence—We and others have demonstrated that MANF is secreted in response to ER stress; however, the function of secreted MANF is not known. As a previous study failed to detect a surface binding of iodinated MANF in neurons (6), we sought to examine association of MANF with the plasma membrane under stressed and unstressed conditions utilizing the tools we have developed and characterized. Our approach was to label surface proteins in SH-SY5Y cell lines using the cell-impermeable sulfo-NHS-SS-biotin. Following a streptavidin pulldown to collect surface proteins, levels of the MANF variants were assessed by anti-GFP and anti-MANF immunoblots. We predicted that MANF lacking the RTDL sequence would be found in greater abundance at the cell surface because of its increased secretion. Whereas GFP-MANF-ΔRTDL is detected in greater abundance in the medium (Figs. 3 and 5), we detected only the full-length protein in the biotinylated fraction (Fig. 7*A*). Additionally, culturing cells in serum-free medium, to increase cellular stress, resulted in increased MANF (endogenous, untagged, and GFP-tagged) observed at the cell surface, but we still did not detect GFP-MANF-ΔRTDL (Fig. 7*A*, *bottom panel*). To further explore the ability of the RTDL sequence to contribute to membrane association, we next examined surface association of SP-GFP-RTDL compared with SP-GFP (Fig. 7*B*). Again, we observed membrane association only for the RTDL-containing proteins (Fig. 7*B*, compare *lanes 9* and *10* with *11* and *12* for GFP immunoblot). We detected a minimal amount of intracellular labeling under the conditions used, as low levels of actin were detected in the surface fraction (Fig. 7, *A* and *B*, *actin immunoblots*); however, the comparatively high levels of MANF observed in the surface fraction support its surface association in SH-SY5Y cells. These results suggest an RTDL-dependent association of MANF with the plasma membrane.

KDELRs at the Cell Surface Increase after Tg-induced ER Stress—Our surface binding experiments suggest that RTDL is important for MANF surface binding, and we hypothesized that KDELRs at the cell surface may mediate this interaction. However, previous studies with these receptors have not indicated any localization to the plasma membrane. To test for surface localization of KDELRs in our SH-SY5Y cell model, we transiently transfected Myc-FLAG-tagged KDELRs and collected surface proteins using Sulfo-NHS-SS-biotin. By this approach, we detected the presence of KDELRs in the cell surface fraction (Fig. 8*A*). Next, we treated the cells for 5 h with Tg and compared the levels of each receptor variant under stressed conditions to standard growth conditions (Fig. 8*B*). We observed an increase in KDELRs at the surface following ER stress induction. Analysis of total cellular KDELR levels revealed a minimal effect of Tg, suggesting the increase in surface levels was not due to an increase in receptor expression (Fig. 8*B*, *WCL*). To further investigate the temporal response of KDELR accumulation at the cell surface, we examined surface KDELR1-Myc-FLAG after 0.5, 2.5, and 5 h of Tg treatment (Fig.

FIGURE 4. **GFP-MANF-ΔRTDL localizes to the ER and Golgi.** *A,* SH-SY5Y cells expressing GFP-MANF-ΔRTDL were examined by confocal microscopy for MANF localization. Cells were transfected with DsRed-ER for 24 h (*top panel*). The Golgi apparatus was immunostained with an RCAS1 antibody (*lower panel*). *B,* SP-GFP is detected in the Golgi. Subcellular colocalization of SP-GFP with the Golgi was examined as described in *A*. *C,* BFA treatment induces the intracellular accumulation of GFP-MANF-ΔRTDL. SH-SY5Y cells were transiently transfected with GFP-MANF or GFP-MANF-ΔRTDL for 24 h. Cells were treated with brefeldin A (50 ng/ml) or ethanol (vehicle) for 3 or 8 h. The effect of BFA was captured by returning to the same field of the well using Nikon Elements AR software and a Prior Proscan III stage. Acquisition settings were constant for all images. *D,* intracellular accumulation of GFP-MANF-ΔRTDL is induced by inhibiting ER to Golgi transport with BFA. GFP-MANF-ΔRTDL was assessed by confocal microscopy after 8 h of BFA (50 ng/ml) or ethanol (vehicle) treatment. Stable SH-SY5Y-GFP-MANF-ΔRTDL cells were transiently transfected with DsRed-ER for 24 h before treatments. Confocal acquisition settings were identical for each group. *Scale bars* on micrographs = 10 μm.

8*C*). Again, we detected an increase in KDELR1 at the cell surface with Tg, with elevated levels detectable after 2.5 h of treatment.

Peptides with Affinity for KDELRs Can Competitively Inhibit MANF Binding at the Cell Surface—Although the presence of the KDELRs at the plasma membrane is consistent with an RTDL-dependent MANF interaction at this site, we sought to further explore MANF surface binding in rat primary cortical neurons. Initially, we examined surface binding of endogenous MANF in these cells using NHS-SS-biotin and detected a significant quantity of MANF in the surface fraction (Fig. 9*A*). Additionally, following 24 h of Tg treatment, we detected an increase in MANF in both the intracellular (Fig. 9*A*, *input lanes*) and surface fractions, with maximal surface levels observed at 10 nM Tg. Next, to test for an association of MANF with KDELRs, our approach was to incubate the cells with two short peptides as follows: YTSEKDEL, a peptide known to interact with the KDEL receptor (27), and ASARTDL, corresponding to the final seven amino acids of MANF (Fig. 9*B*). We predicted that an association between MANF and the KDELR(s) at the cell surface would be inhibited using either of these peptides. After 3 h of incubation with either YTSEKDEL or ASARTDL, a



dose-dependent reduction in surface MANF was observed, suggesting a KDELR-mediated association of MANF with the plasma membrane (Fig. 9*B*). A similar effect on MANF binding was not observed in the presence of the scrambled peptide DRATSAL (Fig. 9*C*).

KDELR1 Overexpression in HEK293 Cells Increases Surface MANF—To further explore the relationship between KDELRs and MANF, we examined the effect of KDELR1 overexpression on surface MANF levels (Fig. 10). HEK293 cells were utilized in these experiments due to the increased transfection efficiency compared with SH-SY5Y cells. Following KDELR1 overexpression, we observed an increase in surface MANF, for both the endogenous and overexpressed proteins. These results are consistent with KDELRs modulating the surface binding of MANF.

DISCUSSION

We created a set of stable SH-SY5Y cell lines expressing MANF variants to study the regulation of its intracellular trafficking. GFP-tagged MANF is localized to the ER and is sensitive to ER stress induction (Figs. 2 and 5). Removal of the C-terminal RTDL sequence increased secretion of the protein, consistent with the bulk flow of the MANF-ΔRTDL protein through the secretory pathway and a loss of retrograde trafficking from the Golgi to ER. These findings are consistent with recent reports, which show that removal of the RTDL sequence increases MANF secretion in non-neuronal cell types (11, 14). We further examined the contribution of the RTDL sequence, independent of additional MANF sequences, using GFP constructs containing only the signal peptide and seven C-terminal amino acids, ASARTDL. Localization experiments revealed striking similarities to the full-length MANF protein, suggesting that the RTDL sequence is a functional ER retention signal and likely plays a large role in regulating MANF's distribution in the cell.

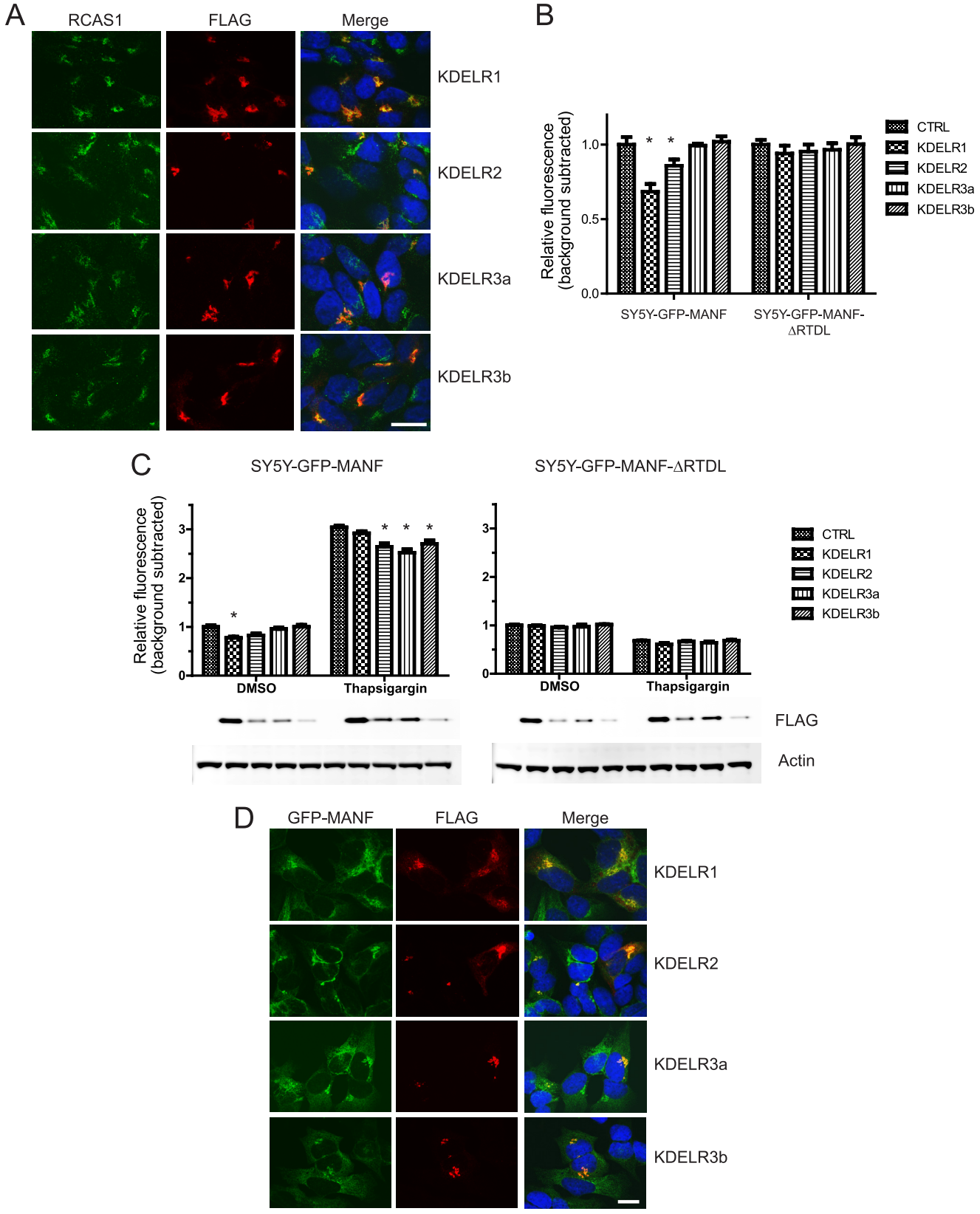
The robust secretory response of MANF to Tg-induced ER stress (Fig. 4*C*), but not tunicamycin or DTT, is consistent with previous *in vitro* observations. Enhanced protein secretion is not a general cellular response to ER stress, and a reduction in overall secretory capacity was previously reported in cells treated with Tg (28). The inhibitory effects of thapsigargin on secretion were also observed in our study, with decreased secretion for both GFP-MANF-ΔRTDL and SP-GFP (Fig. 4*C*). Alternatively, the rapid and robust secretion of full-length MANF is in agreement with a model where MANF is a sensor, and possibly a signaling molecule, in the ER stress-response pathway. We also observed Tg-induced secretion of SP-GFP-RTDL, indicating a responsiveness of the C-terminal sequence to ER stress, independent of additional MANF sequences. This differs from the recently reported results from Glembotski *et al.*, which found the RTDL sequence is not required for the secretory response of MANF to Tg-induced ER stress in HeLa and cardiomyocytes (11). In these cells, MANF secretion was shown to be regulated by a calcium-dependent interaction with GRP78 (BiP) in the ER. This apparent contradiction remains to be resolved. Given the differential levels of KDELR isoforms and their different effects on the Tg-induced secretion of GFP-MANF (Fig. 6), the KDELR composition in these different cell types may provide insight into the observed differences in the role of the RTDL sequence regulating MANF release from the ER.

KDELR overexpression reduced GFP-MANF secretion but had no effect on GFP-MANF-ΔRTDL, supporting a direct interaction between the receptor and the C-terminal sequence of MANF (Fig. 6*B*). Previous studies have observed global inhibitory effects on secretion, a BFA-like phenotype, following KDELR overexpression (29); this is unlikely in our experiments due to the lack of a KDEL receptor effect on GFP-MANF-ΔRTDL secretion (Fig. 6*B*). We also observed KDEL receptor isoform-dependent differences in MANF secretion under stressed and unstressed conditions, supporting the hypothesis that the paralogs have specialized functions. These experiments were done by transfecting the KDELR expression vectors into cell lines that already stably expressed the MANF variants. Because the transfection efficiency was ~20–30%, our results may underestimate the full effect of the KDELRs. As indicated above, we also observed differential expression of the KDELR isoforms in SH-SY5Y cells (Fig. 6*C*, *Western blots*). There was no apparent correlation between the KDELR expression level and its effect on MANF secretion, because the receptor with highest expression (KDELR1) demonstrated no significant effect on secretion following Tg treatment (Fig. 6*C*). As all of the KDELR isoforms were cloned into the same expression vec-

FIGURE 5. **RTDL sequence is required for secretory response to thapsigargin.** *A,* GFP-MANF intracellular fluorescence decreases following Tg treatment. SH-SY5Y cells were treated with 300 nM Tg or vehicle control for 5 h. Fluorescent images were captured using a FITC filter and identical exposure settings. *B,* GFP-MANF secretion in response to Tg requires the RTDL sequence. SY5Y-GFP-MANF and SY5Y-GFP-MANF-ΔRTDL cells were grown overnight in low serum (1.5% BGS) and given fresh medium with DMSO or 300 nM Tg ($t = 0$). Background subtracted (calculated in wells containing media only) fluorescence measurements were made at 1, 2.5, and 5 h, and values were normalized to the DMSO control at $t = 1$ h for each cell line (mean ± S.D.; $n = 3$). Two-factor analysis of variation for GFP-MANF showed a significant main effect for the treatment factor, $F(1,12) = 268.8$, $p < 0.0001$; significant main effect for the time factor, $F(2,12) = 45.86$, $p < 0.0001$; and a significant interaction between treatment and time, $F(2,12) = 62.8$, $p < 0.0001$. For GFP-MANF-ΔRTDL, there was a significant main effect for treatment factor, $F(1,12) = 12.44$, $p < 0.01$; significant effect for time factor, $F(2,12) = 51.8$, $p < 0.0001$; and no significant interaction between treatment and time, $F(2,12) = 3.53$, $p < 0.07$. Importantly, the direction of the effect for the two MANF variants was opposite, with increased secretion for GFP-MANF and decreased secretion for GFP-MANF-ΔRTDL. *C,* secretion of GFP-MANF is not observed in response to tunicamycin. SY5Y-GFP-MANF and SY5Y-GFP-MANF-ΔRTDL cells were treated with the indicated doses of tunicamycin (or DMSO vehicle control) for 5 h. Medium was collected and measured for fluorescence. Mean background-subtracted fluorescence measurements ± S.D. ($n = 3$) are presented. Background fluorescence was measured in media collected from an equal number of parental SH-SY5Y cells. *D,* appending RTDL to the C terminus of GFP is sufficient to confer a secretory response to Tg. Stable SH-SY5Y variants were grown overnight in low serum and given fresh medium containing DMSO or 300 nM Tg for 5 h. Background-subtracted fluorescence measurements (mean ± S.D.; $n = 4$) are presented relative to the SY5Y-GFP-MANF cell line treated with vehicle control. Fold changes (Tg *versus* vehicle, two-tailed *t* test) were as follows: GFP-MANF (6.6 ± 0.13, $p < 0.0001$); SP-GFP-RTDL (5.9 ± 0.20, $p < 0.0001$); GFP-MANF-ΔRTDL (0.78 ± 0.04, $p < 0.01$), and SP-GFP (0.73 ± 0.09, $p < 0.01$). *E,* immunoprecipitation and Western blot analysis confirms increased secretion of RTDL-containing variants in response to Tg. SH-SY5Y stable cell lines were treated for 5 h with 300 nM Tg, and cell culture supernatant was immunoprecipitated for GFP. Samples were immunoblotted for GFP and actin. *I* = intracellular protein (50% input), and *S* = secreted protein eluted from anti-GFP beads. *, $p < 0.01$.

tor with identical promoters, differences in expression would suggest that the proteins are differentially regulated by a post-transcriptional mechanism in SH-SY5Y cells.

Unexpectedly, we detected an RTDL-dependent association of MANF with the surface of SH-SY5Y cells. The importance of this sequence was further confirmed by competition experi-



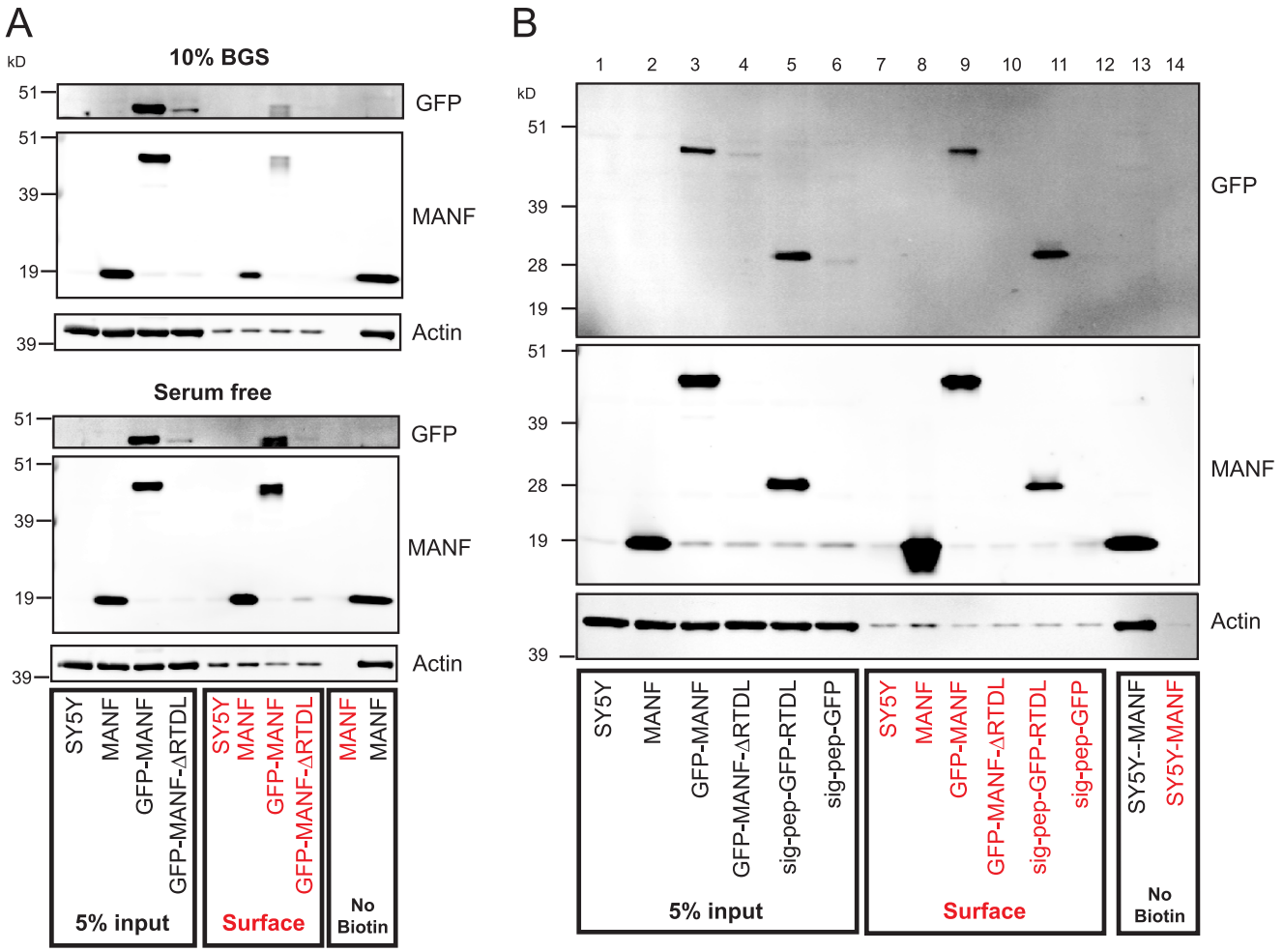


FIGURE 7. **MANF association with the surface of SH-SY5Y cells is RTDL-dependent.** *A,* SH-SY5Y cells were grown in standard culture medium or serum-free medium for 16 h, and biotinylation was performed as described under "Experimental Procedures" using Sulfo-NHS-SS-Biotin and streptavidin-conjugated beads. Whole cell lysates corresponding to 5% of input were also analyzed. Immunoblotting for actin was performed as a control for nonspecific surface labeling. *B,* ASARTDL sequence of MANF is necessary and sufficient for membrane association. SH-SY5Y stable cell lines, including SP-GFP-RTDL and SP-GFP, were grown in low serum medium (1.5% BGS) for 16 h and treated with 300 nM Tg for 5 h. Surface labeling was performed as described in *A*.

ments with rat primary cortical neurons, where treatment with either ASARTDL or YTSEKDEL peptides resulted in decreased levels of surface-biotinylated MANF. The concentration of peptide required to compete with MANF at the surface was much greater than expected, as previous reports showed a 100–200 nM affinity for the YTSEKDEL peptide with purified Golgi membranes (27). This discrepancy could be due to rapid breakdown of the peptides under the cell culture conditions; although we did not measure the stability of our peptides, a previous report indicates many peptides have short half-lives in

FIGURE 6. **KDEL receptor overexpression reduces MANF secretion.** *A,* Myc-FLAG-tagged human KDELRs localize to the Golgi in SH-SY5Y cells. Tagged KDELRs were transiently transfected into SH-SY5Y cells for 48 h, shifted to low serum medium (1.5% BGS) for 16 h, fixed with 4% paraformaldehyde, and immunostained using anti-FLAG and anti-RCAS1 antibodies. *Scale bar,* 10 μm. *B,* KDELR1 and KDELR2 decrease GFP-MANF secretion. SH-SY5Y-GFP-MANF cells were transfected with KDELRs for 48 h and shifted to low serum medium (1.5% BGS) for 16 h. Background-subtracted fluorescence measurements of the medium were normalized to the vector control (*CTRL*) for each cell line (mean $\pm$ S.D., $n = 6$). One-way analysis of variance for GFP-MANF cells showed a significant effect of the KDELRs, $F(4,25) = 72.42$, $p < 0.0001$. Similar analysis for GFP-MANF-ΔRTDL showed no significant effect of the KDELRs, $F(4,25) = 2.377$, $p > 0.05$. The effect of each receptor isoform (in GFP-MANF cells) was compared with the control transfection by Dunnett's multiple comparison test: KDELR1 ($0.68 \pm p < 0.001$), KDELR2 (0.86 ± 0.04, $p < 0.001$), KDELR3a (0.99 ± 0.01, $p > 0.05$), and KDELR3b (1.02 ± 0.04, $p > 0.05$). *C,* KDELR isoforms exhibit nonidentical effects on MANF secretion under Tg-induced ER stress conditions. SH-SY5Y-GFP-MANF and SH-SY5Y-GFP-MANF-ΔRTDL cells were transfected with the KDELRs for 48 h, shifted to low serum (1.5% BGS) medium for 16 h, and treated with 300 nM Tg or DMSO (vehicle control) for 5 h. Background-subtracted fluorescence measurements were normalized to vector control-transfected cells treated with DMSO (mean $\pm$ S.D., $n = 4$). Two-factor analysis of variation for GFP-MANF showed a significant main effect for the receptor factor, $F(4,30) = 10.89$, $p < 0.0001$; significant effect for the treatment factor, $F(1,30) = 3400$, $p < 0.0001$; and a significant interaction between the factors, $F(4,30) = 11.23$, $p < 0.0001$. Similar analysis for GFP-MANF-ΔRTDL showed no significant effect for the receptor factor, $F(4,30) = 2.042$, $p > 0.05$; a significant effect for treatment, $F(1,30) = 527.3$, $p < 0.001$; and no significant interaction between receptor and treatment factors, $F(4,30) = 0.97337$, $p > 0.05$. The effect of each receptor isoform in GFP-MANF cells was compared with control-transfected cells using a Bonferroni multiple comparison post test. For DMSO-treated cells, KDELR1 (0.77 ± 0.05, $p < 0.05$), KDELR2 (0.82 ± 0.09, $p > 0.05$), KDELR3a (0.95 ± 0.06, $p > 0.05$), and KDELR3b (1.00 ± 0.08, $p > 0.05$). For Tg-treated cells, KDELR1 (0.96 ± 0.03, $p > 0.05$), KDELR2 (0.87 ± 0.05, $p < 0.0001$), KDELR3a (0.83 ± 0.05, $p < 0.0001$), and KDELR3b (0.89 ± 0.05, $p < 0.001$). Levels of exogenous KDELRs were assessed by anti-FLAG immunoblots using actin as a loading control. *D,* SH-SY5Y-GFP-MANF cells were plated on glass coverslips and transfected with the KDELR isoforms for 30 h. Cells were shifted to low serum medium (1.5% BGS) for 16 h and treated with 300 nM Tg for 5 h. Cells were fixed and immunostained with anti-FLAG and imaged using a confocal microscope. Nuclei were stained with DAPI. *Scale bars* on micrographs = 10 μm. *, $p < 0.05$.

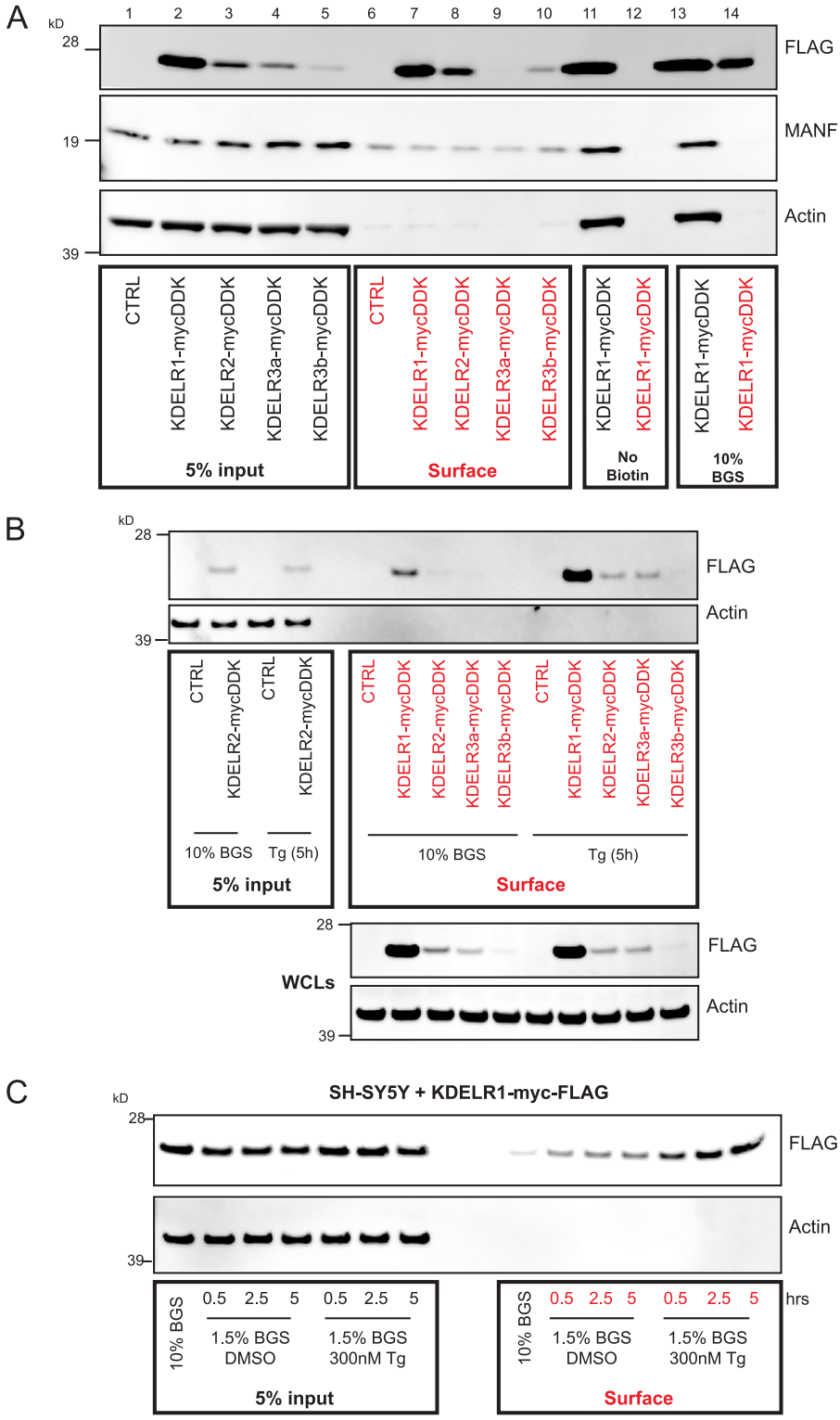


FIGURE 8. **KDELRs at the surface of SH-SY5Y cells increase after ER stress.** *A,* SH-SY5Y cells were transiently transfected with Myc-FLAG-tagged KDELRs for 48 h and given fresh medium containing 1.5% BGS or 10% BGS for 16 h. Surface proteins were labeled with Sulfo-NHS-SS-Biotin and purified with streptavidin beads as described under "Experimental Procedures." Whole cell lysates corresponding to 5% of the surface fraction were also analyzed. *B,* Tg increases surface KDELRs. SH-SY5Y cells treated as described in *A* with the addition of a 5-h treatment period with 300 nM Tg (only cells grown in low serum). Surface proteins were labeled as described above. Whole cell lysate immunoblots are displayed in the *lower panel* and serve to examine total expression of the KDELR variants. *C,* SH-SY5Y cells were transfected with KDELR1-Myc-FLAG for 48 h, given fresh medium (10% BGS or 1.5% BGS) for 16 h, and treated with 300 nM Tg or DMSO for 0.5, 2.5, or 5 h. Surface KDELR1 levels were assessed as described above. *CTRL*, control.

culture (30). Alternatively, the high peptide concentrations may indicate increased affinity between MANF and KDELRs at the cell surface or a cooperative binding effect, where another domain of MANF (such as the N-terminal saposin-like domain) could interact with lipids and enhance overall affinity with the membrane (31). Notably, KDELR interactions with client pro-

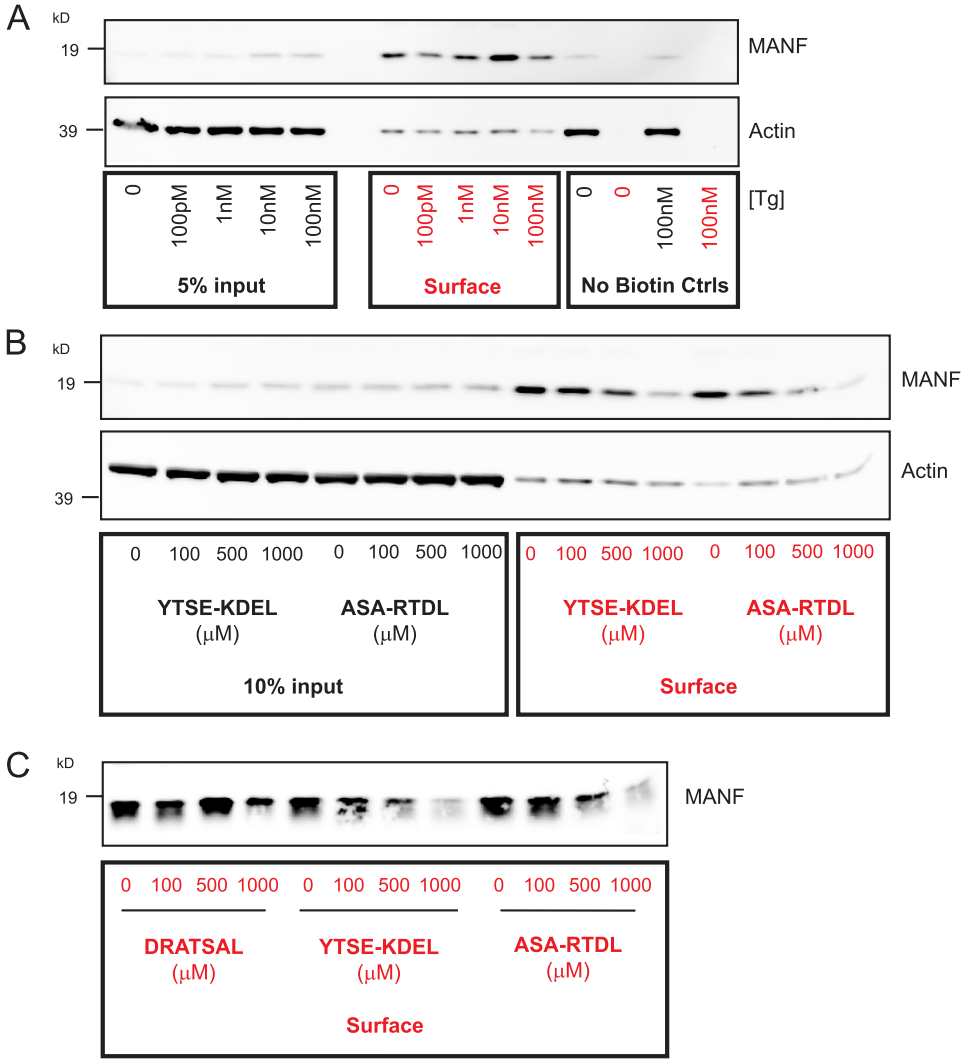


FIGURE 9. **MANF association with the plasma membrane of rat primary cortical neurons is competitively inhibited by KDELR interacting peptides.** *A,* MANF association with the surface of primary cortical neurons is increased by Tg. Primary cortical neuron cultures (DIV14) were treated for 24 h with a range of Tg concentrations. Surface labeling was performed using Sulfo-NHS-SS-Biotin, and labeled proteins were purified with streptavidin-conjugated beads. Whole cell lysates corresponding to 5% of input were also analyzed. Immunoblotting for actin was performed as a control (*Ctrl*) for nonspecific surface labeling. *B,* surface association of MANF is inhibited by YTSEKDEL and ASARTDL peptides. Primary cortical neurons (DIV14) were incubated with peptides for 3 h before biotinylation. Whole cell lysates corresponding to 10% of the surface fraction were also analyzed. *C,* dose-dependent reduction in surface MANF is not observed for cells incubated with a control scrambled peptide DRATSAL. Surface MANF protein was examined as described in *B*.

teins are also pH-dependent, with increased affinity under acidic conditions (27), and it is intriguing to speculate that changes in physiologic conditions that decrease extracellular pH, such as stroke (32), may alter the association of MANF with the plasma membrane to regulate its neurotrophic action against ischemia (4, 5).

The accumulation of surface KDELRs following Tg treatment, with minimal changes in total cellular levels, supports a connection between trafficking the receptor to the surface and ER homeostasis (Fig. 8, *B* and *C*). Surprisingly, we did not observe an increase in MANF binding at cell surface of SH-SY5Y when transiently overexpressing the KDEL receptor isoforms (Fig. 8*A*, *MANF immunoblot*). It is possible that low transfection efficiency in this cell line did not significantly alter the overall number of KDELRs at the cell surface, and our inability to detect endogenous KDELRs with commercial antibodies prevents us from assessing the change in total KDELRs in these experiments (data not shown). We did, however, observe an increase in surface MANF when KDELR1 was transiently overexpressed in HEK293 cells supporting the ability of KDELRs to modulate cell surface binding of MANF (Fig. 10).

Notably, the RTDL-dependent interaction of MANF with the surface may also be modulated by additional KDELR-like proteins. A recent report identified multiple proteins that share structural characteristics with KDELRs (33), and we propose that these are additional candidates that may modulate MANF binding at the plasma membrane. Further studies investigating the localization and functions of these KDELR-like proteins may provide insight into their potential interactions with the MANF RTDL sequence.

Our finding that KDELRs are present at the plasma membrane in SH-SY5Y and HEK293 cells has implications beyond MANF binding. Several toxins contain a KDEL-like sequence that contributes to toxicity, including *Pseudomonas* exotoxin A

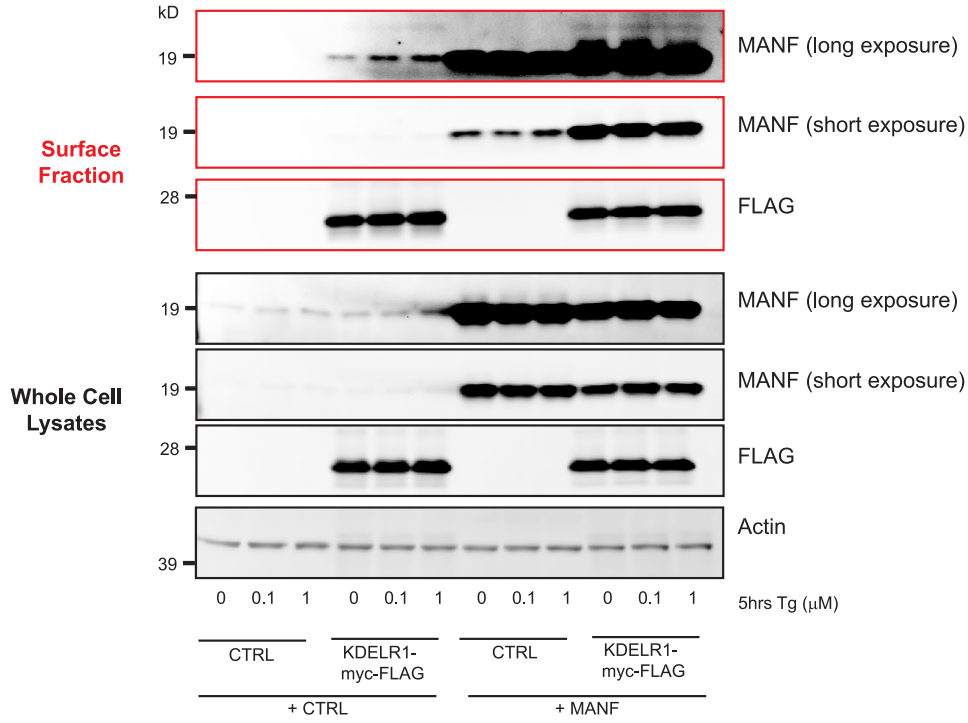


FIGURE 10. **KDELR1 overexpression increases surface levels of endogenous and overexpressed MANF.** HEK293 cells were transfected with pCMV6-KDELR1, pLenti6.3-MANF, and their corresponding control (*CTRL*) plasmids. Cells were incubated for 48 h, treated with 0, 0.1, or 1 μM thapsigargin for 5 h, and biotinylated to label surface proteins. Surface fractions were collected with streptavidin pulldowns, and protein levels were examined by Western blot.

(REDL) and cholera A (KDEL) (34, 35). It is postulated that this sequence increases the toxicity by interacting with the KDELR at the Golgi (after endocytosis) and enhancing toxin access to the ER. Based on our results, it is possible that KDELRs at the membrane may facilitate binding and subsequent translocation to the ER. Although this model is currently purely speculative, it raises intriguing possibilities for preventing toxicity associated with these proteins.

In addition to the retrieval function of KDELRs, a recent report demonstrated a signaling function via a G-protein-coupled receptor-like fold in the KDEL receptors (36). The KDELR has also been shown to modulate activity of the p38 mitogen-activated protein kinases (MAPK and c-Jun N-terminal kinases (JNKs)), which are important critical regulators of cell differentiation and survival (37). KDELRs are also implicated in regulating autophagy, where overexpression of KDELR promoted clearance of neurodegenerative disease-related proteins in cell culture (38). It is therefore plausible that KDEL-related activation of signaling pathways plays a role in promoting survival against neurodegenerative diseases such as stroke and Parkinson disease, and investigating whether MANF alters these pathways (intra- or extracellularly) is of interest.

MANF was identified as a secreted protein from a cell line of astrocyte origin (1); however, subsequent studies found that neurons express the highest levels of MANF in the brain (12, 39). MANF is also expressed in non-neuronal tissue, with widespread distribution and the highest expression generally found in tissues with a large secretory function, *e.g.* testis and salivary gland (12). It is possible that the interaction between MANF and KDELRs is tightly coupled to the secretory homeostasis of cells. Interestingly, a recent genomic study in *Drosophila* revealed perturbations in membrane trafficking in mutants lacking MANF, further implicating a potential role for MANF in protein trafficking pathways (40). In sum, our analyses demonstrate the importance of the C-terminal RTDL sequence in MANF trafficking and response to ER stress in neuronal cell types. Additionally, we observed that the RTDL sequence of MANF is necessary and sufficient for cell surface binding, possibly involving an interaction with the KDELR (or a KDELR-like molecule). These findings provide insight into the mechanisms of MANF neuroprotection, and may be applicable to understanding its functions in other secretory tissues.

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